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THE ROYAL SOCIETY PUBLISHING

Haemolymph transfusions transfer heritable learned novel odour preferences to naive larvae of *Bicyclus anynana* butterflies

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The mechanisms whereby environmental experiences of parents are transmitted to their offspring to impact their behaviour and fitness are poorly understood. Previously, we showed that naive *Bicyclus anynana* butterfly larvae, whose parents fed on a normal plant feed but coated with a novel odour, inherited an acquired preference towards that odour, which had initially elicited avoidance in the naive parents. Here, we performed simple haemolymph transfusions from odour-fed and control-fed larvae to naive larval recipients. We found that larvae injected with haemolymph from odour-fed donors stopped avoiding the novel odour, and their naive offspring preferred the odour more, compared to the offspring of larvae injected with control haemolymph. These results indicate that factors in the haemolymph, potentially the odour molecule itself, play an important role in odour learning and preference transmission across generations. Furthermore, this mechanism of odour preference inheritance, mediated by the haemolymph, bypasses the peripheral odour-sensing mechanisms taking place in the antennae, mouthparts or legs, and may mediate food plant switching and diversification in Lepidoptera or more broadly across insects.

1. Introduction

Parental exposure to a novel stimulus or experience can influence the morphology, physiology or behaviour of their offspring. Such inheritance of acquired traits includes the development of a defensive taller helmet phenotype in water fleas [1,2], resistance to heavy metal toxicity in nematode worms [3], increased immunity against pathogens in *Galleria* moths [4], repulsive behaviour towards a scent in pea aphids and mice [5,6] and preference for a novel odour or pheromone in fruit flies and butterflies [7–9]. All these studies show that the environment can influence an organism's phenotype and that of their offspring. However, the factors that are inherited are still illusive.

When organisms inherit a behavioural preference from their parents, this means that such a preference, expressed in the somatic cells of the central or peripheral nervous system of the parent, is transferred onto their germline (or accessory fluid cells) before being transmitted to their offspring. One vehicle for such transfer is the haemolymph, which circulates and is in direct contact with all the internal organs and tissues [10]. However, two possible behavioural preference inheritance mechanisms, mediated by haemolymph, should be considered. We argue that one involves the transfer of acquired preference factors from the brain to the germline and later to the offspring to impact offspring behaviour. The other involves the transfer of the same molecular stimulus that produced the behavioural change in the parent also

to the germline, via retention of that stimulus in the haemolymph. While concrete evidence supporting either mechanism is lacking, haemolymph is the likely carrier of factors involved in both mechanisms. Thereby, transfusing it from treated to naive individuals and observing an altered phenotype in the latter, and/or in their offspring, can be a first step in isolating candidate factors that transfer a learned preference across individuals, and/or from parents to offspring.

Previous experiments with haemolymph transfers have shown that haemolymph can indeed transfer a 'memory' of a stimulus from experienced to naive recipients, altering the recipient's phenotype [11–15]. But while these studies show that factors in the haemolymph can regulate immunity, behaviour, wing colour patterns, and longevity in the recipients of transfusions, there are barely any studies that explore the effects of haemolymph transfer across generations. It is, thus, still unclear whether behavioural preferences acquired by a parental generation can be transferred to the offspring via haemolymph transfusions (HT).

Bicyclus anynana is a subtropical model nymphalid butterfly that can learn preferences for novel visual cues [16,17] and transmit learned chemical cues to their offspring [8,18]. In previous studies, we showed that larvae that fed on an innate aversive odour, acquired a novel preference for that odour and produced naive offspring that inherited that odour preference [8,19].

In this study, we investigate if the haemolymph of *B. anynana* larvae contains factors that can transfer learned odour preferences from odour-experienced to naive larvae, and subsequently also to their offspring, via simple HT. We fed larvae with odour-coated or control leaves throughout their larval stage. We then transfused haemolymph from each of these larval groups to naive recipients. These recipients were tested for their odour preferences pre- and post-HT. Once the recipient larvae became adults, they mated within each group, and their naive larval offspring were tested for odour preferences. We hypothesized that factors contained in the haemolymph, either the odour itself or molecules produced in the brain downstream of odour feeding, can alter the odour preferences of both the recipient larvae and their offspring.

2. Material and methods

(a) Animal husbandry

B. anynana were reared in a climate-controlled room at 27°C, 60% humidity, and 12 : 12 h light: dark photoperiod. Butterflies were fed on mashed bananas. Corn leaves were placed in adult cages, and wild-type embryos were collected from them after 3–4 h.

(b) Selecting larvae that showed a majority preference towards control or odour

Once larvae hatched from eggs (day 0), their naive odour preferences were tested using odour choice assays (figure 1*a*; electronic supplementary material), and depending on the choice made, those larvae were assigned to either control or odour treatments. The odour used in this study is isoamyl acetate (IAA), which smells like bananas. Larvae of the control group were fed control-coated leaves and those of the odour group were fed odour-coated leaves (additional details in electronic supplementary material). Each larva was reared in a separate plastic container and tracked individually. The larvae were tested again for their odour choices on days 5, 10, 15 and 20. Only larvae that consistently chose the odour they were reared on (either control or odour), for at least four out of the five times they were tested, were selected as the ones that showed a 'majority preference' (figure 1*b*). These 20-day-old (fifth instar) larvae were used as donors in HT.

(c) Haemolymph transfusions and post-transfusion choice assays

Approximately 6 µl of haemolymph was extracted from donor individuals and injected into recipient larvae (figure 1*c*). Injected recipient larvae were reared individually in plastic cups and were fed on uncoated leaves. Choice assays were performed at 24 h and again at 48 h after the HT. After pupation, the pupae were placed into two mesh group cages, depending on the injection treatment. Once the adult butterflies eclosed, they were allowed to mate within their treatment cage. Adults laid eggs on corn leaves placed in the cages, and eggs were then transferred onto Petri plates. As soon as these offspring larvae hatched, choice assays were performed to determine their naive odour choices (figure 1*c*).

(d) Statistical analyses

For all statistical analyses, only data from larvae that made a choice were used (electronic supplementary material, table S1). The numbers of larvae that did and did not make a choice are given as a dataset (electronic supplementary material, table S2).

(i) Testing for larval odour preferences

To test if the proportion of larvae of each treatment choosing odour over control was significantly different from a random 50–50% choice, we used a chi-squared test of goodness of fit. Larvae were considered to have a 'preference' if their choice deviated significantly from 50%.
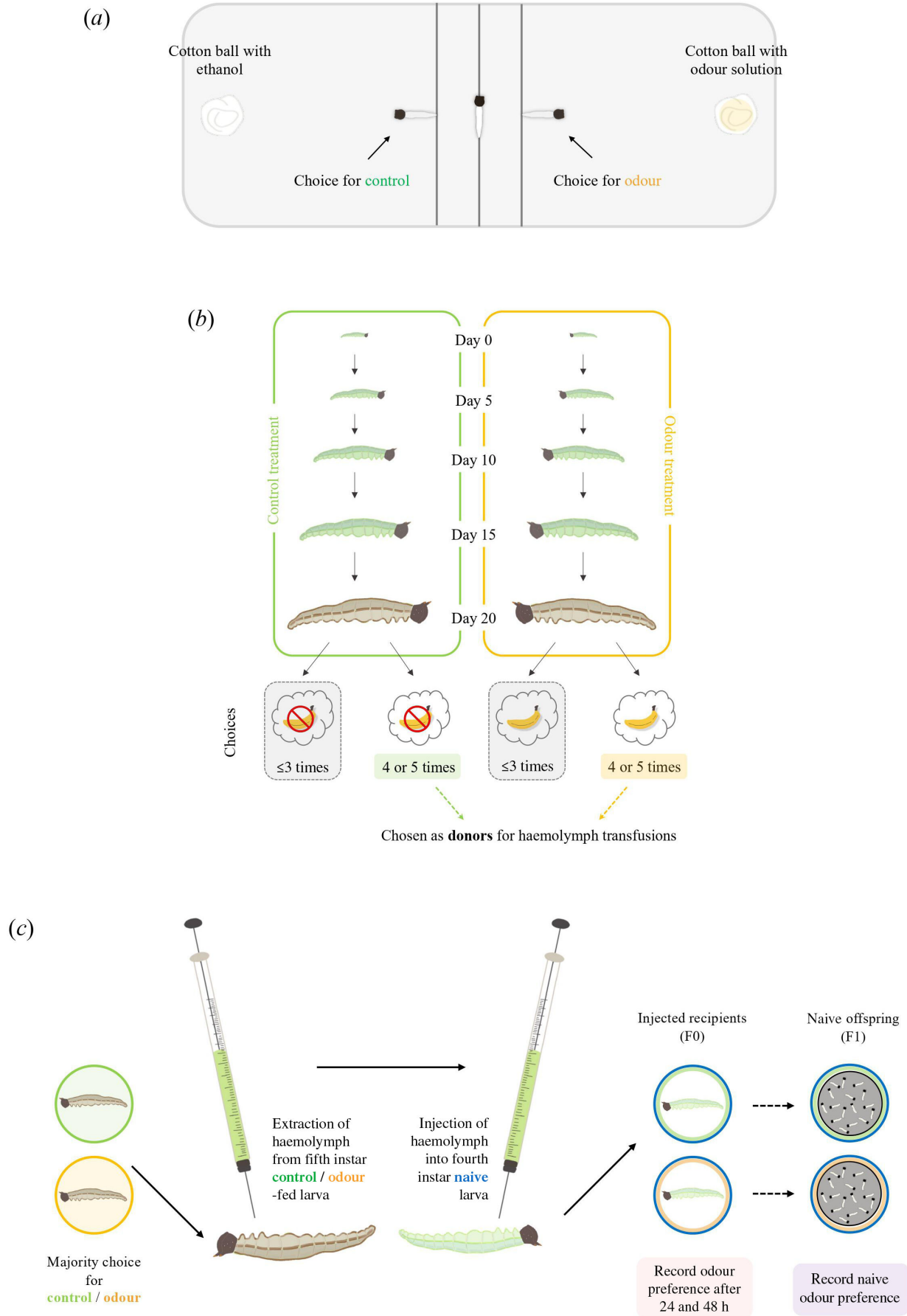
Figure 1. Experimental design. (*a*) *B. anynana* larvae were tested for either control or odour preference using the odour choice assay (additional details in the electronic supplementary material). (*b*) Larvae that showed a consistent preference for the treatment odour they were reared on (at least four out of five choices throughout their development), were chosen as donors for HT. (*c*) Haemolymph from control/odour treatment larvae was extracted and injected into naive larvae. Odour preferences were recorded in the recipient larvae (24 and 48 h after the transfusion) and in their naive offspring.

(ii) Testing for differences in odour choice made by recipient larvae over time and between treatments

We tested the effect of haemolymph type (from control or odour-fed donors) and the time point of odour choice assay (24 and 48 h post-transfusion) on larval odour choice by conducting repeated measures ANOVA on binomial generalized linear mixed-effects model (GLMER). For this specific test, only data from larvae that survived and made a choice until 48 h post-HT were used. In the logit link function, we coded the choice for control as 0, and the choice for odour as 1. We tested for factors that contributed to explaining variation in the response variable using likelihood ratio tests (LRT). Subsequently, we removed the non-significant factor (Pr(>Chisq) > 0.05) from the final model. The choices made by the larvae of both treatments post-HT were compared using pairwise post hoc analysis using least square means and logarithms of odds ratio. The differences in choices made by the larvae pre- and post-HT of each treatment were compared using a two-tailed Fisher exact test of independence.

(iii) Testing the effect of hemolymph transfusion on offspring larvae

The difference in the choices made by the naive offspring larvae between treatments was compared using two-tailed Fisher exact test of independence.

All analyses were performed in the R statistical framework [20], using the packages lsmeans [21], lme4 [22], rcompanion [23], car [24], multcompView [25] and Rmisc [26].

3. Results

(a) Larvae injected with haemolymph from odour-fed donor larvae showed a significant change in their odour preference and so did their offspring

Before HT, naive larvae showed a significant preference for control relative to odour (37% chose odour; $n_{naive} = 112$, $\chi^2 = 8.036$, d.f. = 1, *p*-value = 0.005; figure 2). Twenty-four and 48 h after HT, control (C_{24} or C_{48}) and banana odour-recipient larvae (B_{24} or B_{48}) made random choices, i.e. they showed neither a preference for control nor for odour, when tested at each time point (45% of C_{24} chose odour: $n = 33$, $\chi^2 = 0.273$, d.f. = 1, *p*-value = 0.602; 64% of B_{24} chose odour: $n = 39$, $\chi^2 = 3.103$, d.f. = 1, *p*-value = 0.078; 35% of C_{48} chose odour: $n = 20$, $\chi^2 = 1.8$, d.f. = 1, *p*-value = 0.180; 61% of B_{48} chose odour: $n = 28$, $\chi^2 = 1.286$, d.f. = 1, *p*-value = 0.257; figure 2).

B_{24} and B_{48} larvae showed significantly increased choices for odour relative to pre-injected larvae (Fisher's exact test, 24 h after HT: $n_{naive} = 112$, $nB = 39$, *p*-value = 0.005; 48 h after HT: $n_{naive} = 112$, $nB = 28$, *p*-value = 0.031; figure 2). C_{24} and C_{48} larvae, however, did not show such shifts relative to pre-injected larvae (Fisher's exact test, 24 h after HT: $n_{naive} = 112$, $nC = 33$, *p*-value = 0.418; 48 h after HT: $n_{naive} = 112$, $nC = 20$, *p*-value = 1; figure 2). Odour haemolymph-injected larvae chose the odour significantly more compared to control haemolymph-injected larvae (*post hoc* comparison, B-C: $p = 0.0169$; figure 2, table 1).

Offspring of parents injected with haemolymph from odour-fed larvae (B_{off}), however, showed a significant preference for odour (63% of B_{off} chose odour: $n = 155$, $\chi^2 = 9.813$, d.f. = 1, *p*-value = 0.002; figure 2), whereas offspring of larvae injected with haemolymph from control-fed larvae (C_{off}) made a random choice (42% of C_{off} chose odour: $n = 89$, $\chi^2 = 2.528$, d.f. = 1, *p*-value = 0.118; figure 2). In addition, B_{off} larvae preferred odour significantly more than the C_{off} larvae (Fisher exact test, naive offspring: $nC = 89$, $nB = 155$, *p*-value = 0.002; figure 2).

4. Discussion

In this study, we transfused haemolymph from *B. anynana* larvae fed on different diets, one of them containing a novel odour, to naive larvae to investigate if the haemolymph can influence the odour preferences of recipients and those of their offspring. Our results suggest that the haemolymph of odour-fed larvae contains factors capable of influencing the odour preferences of recipient larvae as well as that of their offspring: it makes them move towards that odour. This study shows the transfer of a learned odour preference across lepidopteran individuals and acquired odour preference across generations using HT.

Changes in phenotype have been previously documented after HT studies [11–15], but our study, documenting a behavioural change, mostly resembles a previous HT study done in mosquitoes [13]. Here, haemolymph from egg-carrying female mosquitos led non-egg-carrying females to halt their host-seeking behaviour [13]. The factors in the haemolymph in these egg-carrying female mosquitoes, likely by-products of internal changes in physiology, was transferred to host females to affect their behaviour. In contrast, our study shows that HT created preferences towards external cues, such as odours, completely bypassing stereotypical odour sensory mechanisms in insects. These mechanisms typically involve the odour molecule binding to gustatory or olfactory receptors in the mouth parts or antennae, and the odour stimulus being transported from the peripheral nervous system to the brain. In addition, our experiment also showed that once dissolved in the haemolymph, odour molecules could have been transported to the germ cells (or seminal fluid), to later influence odour preferences in the offspring that developed from those cells (or were in contact with the seminal fluid as a zygote).

It is still unclear, however, how the encounter with a novel odour induces heritable larval haemolymph chemistry changes. One possibility is the inheritance of the odorant molecules themselves. Odorants from the feed leaves might have directly entered the haemolymph via small openings on the larval cuticle/exoskeleton during respiration, sensory sensilla, or gut.
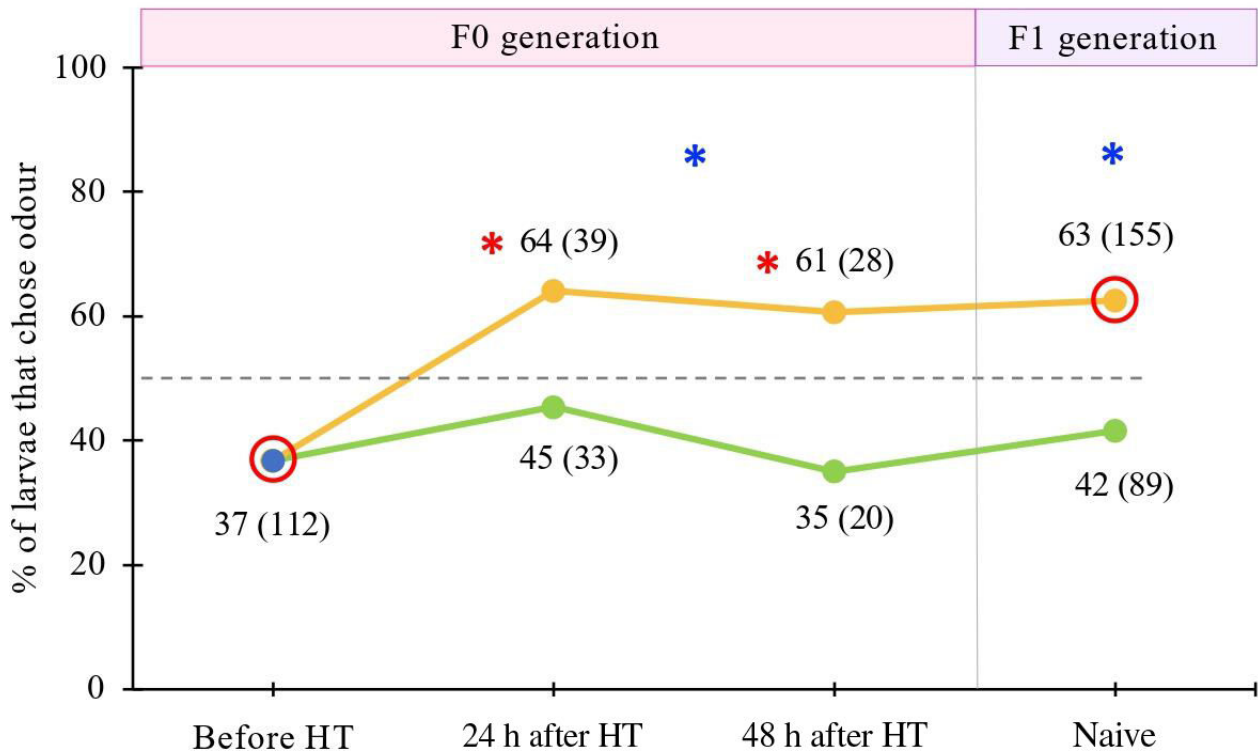
Figure 2. Odour preferences of *B. anynana* larvae before and after HT. Naive recipient larvae (blue dot), with an initial significant avoidance of the novel odour, were later monitored for odour preferences post-transfusion. Green and orange lines represent larvae injected with haemolymph from either control or odour-fed larvae, respectively. Near each point, the percentage values of choice for odour are denoted along with the corresponding total sample sizes (larvae that made a choice) in brackets. Significant preferences (deviation from a random choice) are represented by red circles that outline those specific data points. Red asterisks denote a significantly different choice relative to that made by the larvae before HT. Blue asterisks denote that the choices of both injected recipient and offspring groups are significantly different between treatments (*p-value < 0.05).

Table 1. Summary of repeated measures ANOVA on GLMER (figure 2).

	χ^2	d.f.	*p*-value
odour choice of recipient larvae			
full model variables			
treatment (haemolymph type C or B)	0.076	1	0.783
time point (24 or 48 h post-transfusion)	2.711	1	0.1
treatment × time	0.005	1	0.943
final model variable			
treatment	5.708	1	0.017

Once dissolved in the haemolymph, odour molecules could have been transported to the germ cells, to later influence odour preferences in the offspring that developed from those cells. This haemolymph-mediated mechanism of odour preference development is supported by a study on pigeons where an increased olfactory response was observed upon injection of just the odorant into the blood of recipients [27].

Alternatively, the novel odorant might have induced heritable epigenetic changes in the haemolymph [28–30]. A possible mechanism can start with haemolymph-borne odorants binding to odorant-binding proteins (OBPs) in the antennae, and then being transported to the signal-processing centre of the brain by the circulating haemolymph. These protein-bound odorants might induce DNA methylation marks, histone acetylation or expression of non-coding RNAs in the brain that then travel back to the haemolymph and the germ line. Dias & Ressler suggested that blood-borne odorants might activate olfactory receptors in the germ cells directly and proposed this as a possible mechanism for the inheritance of odour aversion observed in mice [6]. Other mechanisms might include the inheritance of epigenetic factors such as methylation marks on top of specific odour receptors and microRNAs (miRNAs) upon odour-fear conditioning in mice [6,31], starvation-induced small RNAs in nematode worms [32] and piwi-interacting RNAs (piRNAs) that aid in pathogen-avoiding behaviour in worms [33].

Given the diversity of possible mechanisms connected to odour preference/avoidance inheritance, future studies may need to (i) extract, isolate and transfuse different factors from the haemolymph in isolation into naive individuals to observe whether they are responsible for the development of an odour preference, and (ii) explore the epigenetic factors involved in odour learning and inheritance of learned odour preferences. Future studies with *B. anynana* larvae can also be improved by making the choice arena Y-shaped and provided with continuous airflow, to accentuate the difference between odour choices.

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